Applied and Environmental Microbiology, Feb. 2011, p. 1107–1110 Vol. 77, No. 3
0099-2240/11/$12.00 doi:10.1128/AEM.01808-10

The *yctCBA* Operon of *Yersinia ruckeri*, Involved in *In Vivo* Citrate Uptake, Is Not Required for Virulence▽

Roberto Navais, Jessica Méndez, Pilar Reimundo, David Pérez-Pascual, Esther Gómez, and José A. Guijarro*

Área de Microbiología, Departamento de Biología Funcional, Facultad de Medicina, IUBA, Universidad de Oviedo, 33006 Oviedo, Asturias, Spain

Received 29 July 2010/Accepted 23 November 2010

A three-gene operon, named *yctCBA* (*Yersinia* citrate transporter), induced by citrate and repressed by glucose was identified from a previously selected *in vivo*-induced (*ivi*) clone in the fish pathogen *Yersinia ruckeri*. Interestingly, despite being an *ivi* clone, the drastic growth reduction of the *yctC* mutant in the presence of citrate, and the relatively high content of this compound in rainbow trout serum, the operon was not required for virulence.

Yersinia ruckeri is the etiologic agent of enteric redmouth disease (ERM), one of the most widespread diseases in the aquaculture of salmonids (4, 26). Despite the existence of a reasonably effective vaccine that has been administrated for a long time, outbreaks still occur in areas where ERM is endemic. Moreover, the composition of that commercial vaccine does not confer protection against a new biogroup recently isolated in different countries (2, 3, 16). The pathogenic mechanisms of this bacterium are still not very well understood. It has been suggested that extracellular enzymes are related with virulence (23). In this respect, Fernández et al. (15) demonstrated the involvement of Yrp1, a serralysin-type protease, in virulence, but lipolytic activity does not seem to be required for virulence (11). Analysis of a signature-tagged mutagenesis *Y. ruckeri* library (6) identified a high-affinity zinc transporter ZnuABC (8) and the UvrY response regulator of the BarA-UvrY two-component system (7) as virulence factors. In another study, Fernández et al. (12, 13) used an *in vivo* expression technology (IVET) system, 14 *in vivo*-induced genetic loci (*ivi* genes) were selected, some of which have been proven to participate in virulence, such as an iron uptake mechanism via the siderophore ruckerbactin (12), the YhlA hemolysin/cytolysin (14), and a putative type IV secretion system encoded by the *traHIJKCLMN* operon (18). One of the previously selected *ivi* clones named *Y. ruckeri* 150R*iviIX*, contained a partial open reading frame (ORF) that encoded a protein that has significant identity with the TctC protein of the tripartite tricarboxylate transporter family (30). Tricarboxylic acids, particularly citrate, are present in many natural environments, such as fruits, vegetables, milk, and human serum. Characterization and study of the biochemistry and regulatory properties of different citrate transport systems in bacteria have been carried out (5, 10, 30). Interestingly, in plant-pathogenic bacteria, such as *Pectobacterium atrosepticum* (27) and *Xanthomonas campestris* (25), the involvement of citrate uptake systems in virulence has been described.

This study describes the identification in *Y. ruckeri* of the *yctCBA* operon, which was induced by citrate and rainbow trout serum (RTS). Despite being an *ivi* operon, it was not involved in virulence, since a *yctC* mutant was found to be as virulent as the parental strain.

Y. ruckeri 150R, a rifampin-resistant derivative of *Y. ruckeri* 150 strain (14), isolated during naturally occurring outbreaks of ERM at a Danish fish farm (kindly provided by J. L. Larsen, University of Frederiksberg) was used in this work. The strain identification was verified using phenotypic tests (i.e., API 20E) and molecular techniques, including PCR specifically for *Y. ruckeri* (9). Stock cultures were kept in nutrient broth (NB) containing 25% (vol/vol) glycerol at −70°C. Bacteria were routinely grown on nutrient broth agar (NA) plates at 18°C. Pathogenicity was determined by the presence of characteristic external and internal signs of disease associated with ERM (4) and confirmed by 50% lethal dose (LD_{50}) as well as by kinetic challenge experiments.

Analysis of the *ivi* clone. DNA sequencing of *Y. ruckeri* 150R*iviIX* was carried out by the method of Fernández et al. (12). The analysis of the sequence revealed the presence of a partial open reading frame of 424 bp encoding a protein with high identity to TctC, a protein from *Salmonella enterica* serovar Typhimurium involved in the transport of tricarboxylic substrates. The sequencing of the region adjacent to this genetic locus allowed the identification of the *yctCBA* genes flanked by a putative promoter sequence and a rho-independent transcriptional terminator (Fig. 1). Analysis of ORFs using Blastx, PSORTb, SignalP, and SOSUI programs revealed the characteristics of the translational products. The first ORF consisted of 981 bp and encodes a protein of 326 amino acids that shows a high degree of identity to TctC from *S. enterica* serovar Typhimurium (79%) (24), a putative tricarboxylate transport protein from *Serratia proteamaculans* (86%) (GenBank accession no. YP_001479568) and BctC, the corresponding protein from *Bordetella pertussis* (62%) (1). Due to its similarity, the gene was named *yctC*; the predicted protein carries a signal peptide of 23 amino

* Corresponding author. Mailing address: Área de Microbiología, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Asturias, Spain. Phone: 34985104218. Fax: 34985103534. E-mail: jaga@fq.uniovi.es.

▽ Published ahead of print on 3 December 2010.

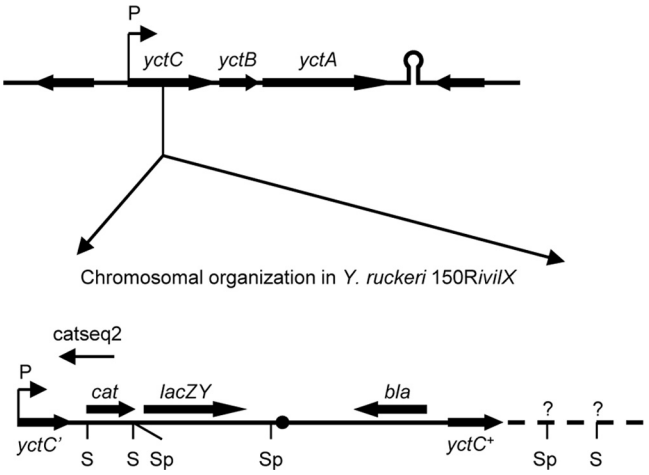

FIG. 1. Chromosomal organization of the region containing the *yctCBA* operon in *Y. ruckeri* 150R. The direction of transcription is indicated by arrows. The arrangement of the transcriptional fusion between *ytcC* and the promoterless genes *lacZY* in the *Y. ruckeri* 150R*iviIX* strain is shown below the map. The putative promoter (P) selected by IVET is indicated, as well as the catseq2 oligonucleotide used to sequence the fragment adjacent to the pIVET8 integration site. The black circle represents the pIVET8 replication origin. S, SalI site; Sp, SphI site; *yctC'*, initial 424 bp of the *yctC* gene; *yctC*$^+$, complete copy of the *yctC* gene; *cat*, chloramphenicol acetyltransferase gene (promoterless); *lacZY*, genes for lactose fermentation (promoterless); *bla*, ampicillin resistance gene.

acids, and it was suggested that YctC would probably be a periplasmic protein. A second ORF, of 432 bp, was found 16 bp downstream of *yctC*. The product of this gene had high sequence identity with TctB from *S. enterica* serovar Typhimurium (66%) (28, 29) and a hypothetical protein from *Serratia odorifera* (76%) (GenBank accession no. ZP_06191120) and had substantially lower identity with BctB from *B. pertussis* (35%) (1). The gene was named *yctB*, and the deduced amino acid sequence is predicted to be a cytoplasmic membrane protein with 5 transmembrane regions. A third ORF (1,518 bp), named *yctA*, was found 8 bp downstream of *yctB*. It encoded a protein of 505 amino acids which showed high identity with TctA from *S. enterica* serovar Typhimurium (87%) (28, 29), putative proteins from *S. proteamaculans* (94%) (GenBank accession no. YP_001479570) and *S. odorifera* (94%) (GenBank accession no. ZP_06191121), and BctA from *B. pertussis* (59%) (1). YctA is predicted to be a cytoplasmic membrane protein, with 12 transmembrane helices and a signal anchor. According to the identities and the intergenic regions, the genes form a *yctCBA* operon encoding three proteins involved in the transport of tricarboxylic acid cycle intermediates into the cell. In *S. enterica* serovar Typhimurium, three different citrate utilization systems (TctI, TctII, and TctIII) have been described, but only TctI, corresponding to the TctCBA system, has been well characterized (28, 29). A similar operon is present in *Corynebacterium glutamicum* (21) and *Bordetella pertussis* (1).

Citrate induces the *yctCBA* promoter. The *Y. ruckeri* 150R*iviIX* strain contains a transcriptional fusion between the *yctCBA* promoter and the *lacZY* genes (Fig. 1), which was used to study the regulation of the *yctCBA* operon. For promoter expression studies, 250-ml flasks containing 20 ml of M9 medium supplemented with 0.2% (wt/vol) Casamino Acids (M9C) were inoculated with 200 μl of a *Y. ruckeri* 150R*iviIX* overnight culture, followed by incubation in orbital shakers at 250 rpm. Samples of 1 ml from exponential-phase cultures were collected and stored at −20°C. The β-galactosidase activity of the *yctC*::pIVET8 transcriptional fusion was measured in triplicate in three independent experiments by the method of Miller (19), using ONPG (*o*-nitrophenyl-β-D-galactopyranoside) as a substrate. After an analysis of variance test, P values of <0.05 were considered significant. The results showed that both trisodium citrate and trisodium isocitrate (Sigma Chemical Company S.A.) exerted an influence on the transcription levels of the *yctCBA* promoter. Thus, the levels of β-galactosidase activity were 3.5-fold higher when the cells were incubated in medium supplemented with citrate. In fact, maximal induction was obtained at a citrate concentration of 1 mM. In addition, the expression of the *yctCBA* promoter was 2-fold higher at 18°C, a temperature related to the natural infection process, than at 28°C, the optimal growth temperature of this microorganism. A glucose concentration of 0.5% (wt/vol) was enough to turn promoter expression off, this being a clear example of catabolic repression. This phenomenon is not generally observed in all species, given that in the homologous system of *C. glutamicum*, citrate uptake was not under catabolic repression (21).

In order to determine why the *yctCBA* operon, induced by citrate, was selected as an *ivi* clone, rainbow trout serum was obtained from blood samples taken from the caudal vein, collected in BD Vacutainer SST II Advance tubes, and centrifuged for 15 min at 1,300 rpm to separate the serum from the cells. A commercial fetal bovine serum (FBS) (Invitrogen Life Technologies) was also used. *Y. ruckeri* 150R*iviIX* strain was incubated in M9C medium supplemented with 10% (vol/vol) of either RTS or FBS, and β-galactosidase activity was determined in exponential-phase cultures. Increases of 24.25% ± 5.57% and 48.36% ± 7.79% in *yctCBA* promoter expression were obtained for RTS and FBS, respectively. High-performance liquid chromatographic (HPLC) analysis of both sera was performed by the method of González de Llano et al. (17) using an ionic exclusion column ICSep ION-300 (Transgenomic, San Jose, CA) loaded with 100 μl of a serum sample that had previously been diluted 10-fold and filtered (0.45 μm). Citric acid elution occurred at 14 min, and quantification was performed by using a linear regression equation ($R^2 \geq 0.99$) using citric acid at different concentrations as a standard. A clear peak eluting in the same minute as the control citrate was found for RTS and FBS, revealing the presence within each peak of approximately 100 μM and 200 μM citrate, respectively. Therefore, proportional induction was found between the amount of citrate present in each serum sample and the increase in β-galactosidase activity, confirming a dose-related response promoter model. As sera were diluted 10-fold in the M9C medium, the results indicated that citrate concentrations of 10 μM and 20 μM would be enough to induce the *yctCBA* promoter, although it should be remembered that other operon inducers could be present in the sera. All the results clearly established that citrate was present in animal sera in concentrations that would make it useful as a nutrient component. However, there were no growth differences between the wild-type strain and the *yctC* mutant strain when they were incubated using rainbow trout serum as a medium (data not

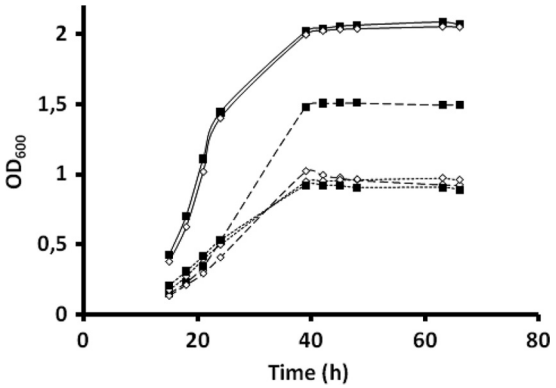

FIG. 2. Growth curves of *Y. ruckeri* 150R and *yctC* mutant strains in different media. Aliquots of overnight cultures were used to inoculate 250-ml flasks containing 20-ml portions of the different media, and cultures of *Y. ruckeri* 150R (■) and *yctC* mutant (◇) were grown at 18°C and 250 rpm in the different media. The media used were M9C medium plus 0.5% glucose (solid black line),M9C medium plus 25 mM trisodium citrate (broken line), and M9C medium (dotted line). Growth was monitored by determining the optical density at 600 nm (OD_{600}) of the culture. The values are the averages for three independent experiments.

shown). In the same way, immersion challenge trial kinetic experiments showed that the inability of the *yctC* mutant to use citrate was not relevant to the progression of the infection, since that was similar to that of the wild-type strain (data not shown). It seems that in complex nutrient environments, citrate utilization is not essential for the bacterial growth. Nevertheless, we cannot rule out the possibility that, in some particular environments or conditions of nutrient shortage, the ability to use citrate provides an advantage to this bacterium.

The *yctCBA* operon is required for growth on citrate but does not contribute to the virulence of *Y. ruckeri*. In order to determine the role of this operon in the physiology and virulence of the bacterium, a *yctC* mutant was obtained by homologous recombination between an internal *yctC* fragment and the corresponding sequence in the chromosome. Therefore, a 457-bp internal fragment of the *yctC* gene was amplified by PCR with the primers iviIX-E (5′-ATGA*GAATTC***CGGTTCG CTACTTAATC**-3′; nucleotides 288 to 304, which correspond with the gene sequence, are in boldface type) and iviIX-S (5′-AGGA*GTCGAC***GAAACCCTGTTCTTTGG**-3′; nucleotides 744 to 728, which correspond with the gene sequence, are in boldface type). The 5′ ends of the iviIX-E and iviIX-S primers contained restriction sites (in italics) for EcoRI and SalI, respectively, and four additional bases. The generated amplicon was digested with both enzymes and ligated into the pJP5603 vector (20), previously digested with the same enzymes. The ligation mixture was used to transform electrocompetent cells of *Escherichia coli* S17-1λ*pir*. Selected transformants, containing the plasmid with insert, were used to conjugate with *Y. ruckeri* 150R to obtain the *yctC* mutant. The presence of the mutation was confirmed by Southern blot analysis (data not shown), and the stability of the mutation in the absence of the antibiotic was checked as previously described (18). According to the operon structure, this mutation should be a polar mutation. The growth of the *yctC* mutant strain on M9C medium containing 25 mM sodium citrate was considerably retarded in relation to that of the parental strain (Fig. 2) showing that this mutant had drastic limitation for growth in the presence of citrate as the sole carbon source. Moreover, similar growth curves for both mutant and parental strains were obtained when M9C medium alone and M9C medium supplemented with 0.5% (wt/vol) glucose were used (Fig. 2). This suggested that the YctCBA system is the only citrate transporter under the conditions assayed and that the *yctC*::pJP5603 mutation suppressed citrate utilization.

Once the determining role of YctC for citrate utilization as well as the presence of important citrate levels in the RTS had been defined, we wanted to know whether the YctCBA system was involved in bacterial virulence, given that the selection of the *Y. ruckeri* 150R*iviIX* clone indicated that the gene is specifically expressed in the fish. LD_{50} experiments using the parental and mutant strains were performed by the method of Fernández et al. (15). Briefly, groups of 10 fish (*Oncorhynchus mykiss*) weighing between 10 and 15 g were kept in 60-liter tanks at 18°C ± 1°C. They were challenged by intraperitoneal injection of 0.1-ml portions of serial 10-fold dilutions, and mortality was monitored for up for 7 days. Two independent experiments were carried out, and the LD_{50} determinations were calculated by the method of Reed and Muench (22). All the animal experiments were conducted in accordance with European legislation governing animal welfare and were authorized and supervised by the Animal Experimentation Ethics Committee of Oviedo University. The results indicated that no significant differences were found between the LD_{50}s of the parental strain (3.6×10^5 CFU) and mutant strain (1.9×10^5 CFU). Therefore, the tricarboxylate transporter system YctCBA does not contribute to virulence in rainbow trout.

Despite being selected as an *ivi* clone, it seems that the *yctCBA* operon is involved in nutrition given that citrate was present in RTS and is a compound that could be introduced into the tricarboxylic and glyoxylate cycles. According to the results, the system is not essential for growth *in vivo* and does not seem to represent an advantage for bacterial survival and progression in the host. These results contrast with those obtained in the phytopathogenic bacteria *P. atrosepticum* (27) and *X. campestris* (25) in which citrate uptake is involved in bacterial virulence. Nevertheless, the involvement in virulence of this system should be evaluated in each case in view of the fact that citrate is a component of animal sera.

Nucleotide sequence accession number. The sequences for the genes of the *yctCBA* operon have been deposited in GenBank database under accession number HM991732.

This work was supported by grant AGL2009-07003 from the Spanish Ministry of Sciences and Education (MEC) of Spain. R.N., P.R., D.P.-P., and E.G. were the recipients of grants from the MICINN.

We thank J. L. Martinez for his help in rainbow trout serum extraction and Lucia Fernández for her previous work on IVET.

REFERENCES

1. **Antoine, R., et al.** 2005. The periplasmic binding protein of a tripartite tricarboxylate transporter is involved in signal transduction. J. Mol. Biol. **351:**799–809.
2. **Arias, C. R., et al.** 2007. First report of *Yersinia ruckeri* biotype 2 in the USA. J. Aquat. Anim. Health **19:**35–40.
3. **Austin, D. A., P. A. W. Robertson, and B. Austin.** 2003. Recovery of a new biogroup of *Yersinia ruckeri* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum). Syst. Appl. Microbiol. **26:**127–131.
4. **Austin, B., and D. A. Austin.** 2007. Bacterial fish pathogens: diseases of farmed and wild fish, 4th ed. Springer-Praxis, Godalming, United Kingdom.

5. **Bott, M.** 1997. Anaerobic citrate metabolism and its regulation in enterobacteria. Arch. Microbiol. **167:**78–88.
6. **Dahiya, I., and R. M. Stevenson.** 2010. *Yersinia ruckeri* genes that attenuate survival in rainbow trout (*Oncorhynchus mykiss*) are identified using signature-tagged mutants. Vet. Microbiol. **144:**399–404.
7. **Dahiya, I., and R. M. Stevenson.** 2010. The UvrY response regulator of the BarA-UvrY two-component system contributes to *Yersinia ruckeri* infection of rainbow trout (*Oncorhynchus mykiss*). Arch. Microbiol. **192:**541–547.
8. **Dahiya, I., and R. M. Stevenson.** 2010. The *ZnuABC* operon is important for *Yersinia ruckeri* infections of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish. Dis. **33:**331–340.
9. **del Cerro, A., I. Marquez, and J. A. Guijarro.** 2002. Simultaneous detection of *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, and *Yersinia ruckeri*, three mayor fish pathogens, by multiplex PCR. Appl. Environ. Microbiol. **68:**5177–5180.
10. **Drider, D., S. Bekal, and H. Prévost.** 2004. Genetic organization and expression of citrate permease in lactic acid bacteria. Genet. Mol. Res. **3:**273–281.
11. **Evenhuis, J. P., S. E. Lapatra, D. W. Verner-Jeffreys, I. Dalsgaard, and W. T. J. Welch.** 2009. Identification of flagellar motility genes in *Yersinia ruckeri* by transposon mutagenesis. Appl. Environ. Microbiol. **75:**6630–6633.
12. **Fernández, L., I. Márquez, and J. A. Guijarro.** 2004. Identification of specific *in vivo*-induced (*ivi*) genes in *Yersinia ruckeri* and analysis of ruckerbactin, a catecholate siderophore iron acquisition system. Appl. Environ. Microbiol. **70:**5199–5207.
13. **Fernández, L., J. Méndez, and J. A. Guijarro.** 2007. Molecular virulence mechanisms of the fish pathogen *Yersinia ruckeri*. Vet. Microbiol. **125:**1–10.
14. **Fernández, L., M. Prieto, and J. A. Guijarro.** 2007. The iron and temperature regulated haemolysin YhlA is a virulence factor of *Yersinia ruckeri*. Microbiology **153:**483–489.
15. **Fernández, L., P. Secades, J. R. López, I. Márquez, and J. A. Guijarro.** 2002. Isolation and analysis of a protease gene with an ABC transport system in the fish pathogen *Yersinia ruckeri*: insertional mutagenesis and involvement in virulence. Microbiology **148:**2233–2243.
16. **Fouz, B., C. Zarza, and C. Amaro.** 2006. First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. J. Fish Dis. **29:**339–346.
17. **González de Llano, D., A. Rodriguez, and P. Cuesta.** 1996. Effect of lactic starter cultures on the organic acid composition of milk and cheese during ripening-analysis by HPLC. J. Appl. Microbiol. **80:**570–576.
18. **Méndez, J., et al.** 2009. A chromosomally located *traHIJKCLMN* operon encoding a putative type IV secretion system is involved in the virulence of *Yersinia ruckeri*. Appl. Environ. Microbiol. **75:**937–945.
19. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 354. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
20. **Penfold, R. J., and J. M. Pemberton.** 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene **118:**145–146.
21. **Polen, T., D. Schluesener, A. Poetsch, M. Bott, and V. F. Wendisch.** 2007. Characterization of citrate utilization in *Corynebacterium glutamicum* by transcriptome and proteome analysis. FEMS Microbiol. Lett. **273:**109–119.
22. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
23. **Romalde, J. L., and A. E. Toranzo.** 1993. Pathological activities of *Yersinia ruckeri*, the enteric redmouth (ERM) bacterium. FEMS Microbiol. Lett. **112:**291–300.
24. **Sweet, G. D., C. M. Kay, and W. W. Kay.** 1984. Tricarboxylate-binding proteins of *Salmonella typhimurium*. Purification, crystallization, and physical properties. J. Biol. Chem. **259:**1586–1592.
25. **Tamir-Ariel, D., N. Navon, and S. Burdman.** 2007. Identification of genes in *Xanthomonas campestris* pv. vesicatoria induced during its interaction with tomato. J. Bacteriol. **189:**6359–6371.
26. **Tobback, E., A. Decostere, K. Hermans, F. Haebebrouck, and K. Chiers.** 2007. *Yersinia ruckeri* infections in salmonid fish. J. Fish Dis. **30:**257–268.
27. **Urbany, C., and H. E. Neuhaus.** 2008. Citrate uptake into *Pectobacterium atrosepticum* is critical for bacterial virulence. Mol. Plant Microbe Interact. **21:**547–554.
28. **Widenhorn, K. A., W. Boos, J. M. Somers, and W. W. Kay.** 1988. Cloning and properties of the *Salmonella typhimurium* tricarboxylate transport operon in *Escherichia coli*. J. Bacteriol. **170:**883–888.
29. **Widenhorn, K. A., J. M. Somers, and W. W. Kay.** 1988. Expression of the divergent tricarboxylate transport operon (*tctI*) of *Salmonella typhimurium*. J. Bacteriol. **170:**3223–3227.
30. **Winnen, B., R. N. Hvrup, and M. H. Saier, Jr.** 2003. The tripartite tricarboxylate transporter (TTT) family. Res. Microbiol. **154:**457–465.